Improved functional and histochemical outcomes in l-DOPA plus tolcapone treated VMAT2-deficient mice

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Abstract: Parkinson disease is typically treated with L-3,4-dihydroxyphenylalanine (or levodopa) co-prescribed with concentration stabilizers to prevent undesired motor fluctuations. However, the beneficial role of the chronic combined therapy on disease progression has not been thoroughly explored. We hypothesized that tolcapone, a catechol-O-methyl-transferase inhibitor, co-administered with levodopa may offer beneficial long-term disease-modifying effects through its dopamine stabilization actions. Here, we followed vesicular monoamine transporter 2-deficient and wild-type mice treated twice daily per os with vehicle, levodopa (20 mg/kg), tolcapone (15 mg/kg) or levodopa (12.5 mg/kg) + tolcapone (15 mg/kg) for 17 weeks. We assessed open field, bar test and rotarod performances at baseline and every 4th week thereafter, corresponding to OFF-medication weeks. Finally, we collected coronal sections from the frontal caudate-putamen and determined the reactivity level of dopamine transporter. Vesicular monoamine transporter 2-deficient mice responded positively to chronic levodopa + tolcapone intervention in the bar test during OFF-periods. Neither levodopa nor tolcapone interventions offered significant improvements on their own. Similarly, chronic levodopa + tolcapone intervention was associated with partially rescued dopamine transporter levels, whereas animals treated solely with levodopa or tolcapone did not present this effect. Interestingly, 4-month progression of bar test scores correlated significantly with dopamine-transporter-label density. Overall, we observed a moderate functional and histopathological improvement effect by chronic dopamine replacement when combined with tolcapone in vesicular monoamine transporter 2-deficient mice. Altogether, chronic stabilization of dopamine levels by catechol-O-methyl-transferase inhibition, besides its intended immediate actions, arises as a potential long-term beneficial approach during the progression of Parkinson disease.

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1. Introduction (we don't have a title and an abstract page?)

Oral administration of L-3,4-dihydroxyphenylalanine (levodopa or L-DOPA), the endogenous precursor of dopamine (DA), combined with a DA decarboxylase inhibitor, such as benserazide hydrochloride or carbidopa, is currently the most effective, tolerable, and therefore broadly used treatment for patients with Parkinson disease (PD). However, the use of L-DOPA is burdened with several limitations, including short-time bioavailability of each dose due to extensive peripheral metabolism. In addition, there is the assumption that long-term dopaminergic stimulation with L-DOPA facilitates the occurrence of motor fluctuations with dyskinesia (Connolly and Lang, 2014; Jenner et al., 2011). The latter have been closely related to disease duration, and treatment duration and dosage (Schrag and Quinn, 2000).

Recent recommendations, therefore, suggested to evaluate alternative pharmacotherapies than L-DOPA for treatment initiation in certain patients, to avoid long-term levodopa-related motor complications (Connolly and Lang, 2014). On the other hand, an elegant delayed-start trial in early PD patients recently found similar rates of dyskinesia and L-DOPA-related motor fluctuations at 80 weeks when comparing a group treated with L-DOPA for 80 weeks and another group placebo-treated for 40 weeks followed by 40 weeks of L-DOPA treatments (Verschuur et al., 2019). The authors concluded that L-DOPA treatment ~~duration~~ may not ~~have~~bear a disease-modifying effect, at least in relation to motor fluctuations.

In addition to treatment duration and dosing, the pattern of DA receptor stimulation has been associated with the emergence of dyskinesias: continuous dopaminergic treatment - either with long-acting DA-receptor agonists or continuous L-DOPA

infusions - is related to fewer ~~motor fluctuations during~~dyskinesias and motor fluctuations, as for instance evidenced in a primate PD model ~~on medication~~ (Bibbiani et al., 2005). This finding has driven the search for strategies to reduce pulsatile DA receptor stimulation (Nyholm, 2006; Olanow and Schapira, 2013; Chondrogiorgi et al., 2014). The goal of the STRIDE-PD study was to examine whether adding entacapone, an inhibitor of catechol-O-methyltransferase (COMT), to L-DOPA reduces the development of dyskinesia, as this approach increases L-DOPA's half-life and smoothens its serum concentrations, i.e. leads to a mildly enhanced continuous stimulation of postsynaptic DA receptors (Stocchi and Olanow, 2004; Stocchi et al., 2010). The study was negative, but the authors rightfully discussed the outcome's relation with the study design, as it provided higher L-DOPA dose equivalents in the entacapone group. ~~In fact~~On the other hand, experimental animal models of LIDs (L-DOPA induced dyskinesias) suggest that the loss of nigrostriatal DA neurons may be the cause for or at least contribute to the development of such abnormal involuntary movements (Cenci, 2014). ~~All in all~~Altogether, despite the extensive and negative STRIDE study, it is still unclear whether COMT inhibition during ~~prolonged~~long-term dopaminergic stimulation at bioavailable L-DOPA equivalent doses offers any ~~long-lasting and/or~~ disease-modifying effect in PD, ~~such as~~which would be best reflected by deceleration of nigrostriatal DA denervation, and which in turn~~,~~ might have a beneficial~~n~~ impact on LIDs onset and severity.

Tolcapone, a selective and reversible COMT inhibitor penetrating the blood-brain barrier (Guldberg and Marsden, 1975; Backstrom et al., 1989; Kopin, 1985; Nagatsu and Sawada, 2009a; Kiss et al., 2010), offers a potent and sustained inhibition *in* vivo (Zürcher et al., 1990; Bonifácio et al., 2007). ~~It is widely accepted that central COMT inhibition by tolcapone reduces degradation of L-DOPA to 3-O-methyldopa, thus~~

~~widening its therapeutic window, while further prolonging L-DOPA effectiveness in the brain by reducing DA metabolism to homovanillic acid (Kurth and Adler, 1998; Kaakkola, 2000; Zeuner et al., 2019).~~Besides peripherally inhibiting L-DOPA metabolism, tolcapone further inactivates central COMT and reduces degradation of extrasynaptic or diffused DA to 3-methoxytyramine (Kurth and Adler, 1998; Kaakkola, 2000; Zeuner et al., 2019), thus, stabilizing its bioavailable synaptic concentration and modulating dopaminergic transmission of presynaptic and postsynaptic neurons (Chen et all., 2011). Ultimately, continuous postsynaptic dopaminergic signaling by tempered and stable DA levels will possibly lead to long-term clinical and pathological amelioration. Notwithstanding, tolcapone was associated with increases in liver enzymes in a dose-dependent manner (Longo et al., 2016). Peripheral inhibition of soluble-COMT may be at least in part, the main underlying cause of tolcapone-induced hepatotoxicity: uncoupling of oxidative phosphorylation and the subsequent reduction in mitochondrial energy (Haasio et al., 2002).

~~The cause of L-DOPA-induced motor fluctuations is not entirely understood. Apart from contributions from disturbed neurotransmission, the best available evidence suggests that progressive degeneration of DA-producing cells in the substantia nigra is mandatory (Leta et al., 2019). In parallel, degeneration of dopaminergic terminals along with aberrant processing of exogenously administered L-DOPA lead to imbalanced striatal DA levels, causing motor fluctuations (Niccolini et al., 2015). For instance, the link of dyskinesia with dopaminergic denervation is supported by findings in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) addicts and in rodents with complete nigral lesions (Winkler et al., 2002; Langston, 2017), although these studies lacked a necessary longitudinal perspective. Altogether, there appears to be a strong~~

~~association between the degree of nigrostriatal denervation/neurodegeneration and the expression of dyskinesia (Di Monte et al., 2000).~~

~~Ultimately contributing to neurodegeneration as well, chronic~~Chronic use of L-DOPA generates several toxic molecules ~~(Paul and Borah, 2016),~~ responsible for increased predisposition to ~~vascular disease and~~ accelerated disease progression (Müller, 2011). One such metabolite is homocysteine (Hcy), the level of which increases in the plasma of PD patients ~~ultimately generating~~composes a high risk for vascular diseases and dementia (Kocer et al., 2016). Furthermore, Hcy exerts a neurotoxic action and may participate in the mechanisms of neurodegeneration, such as DNA damage, excitotoxicity, oxidative stress ~~and neuronal degeneration (Bhattacharjee and Borah, ~~, calcium accumulation, and apoptosis (Zoccolella et al., 2006a). Several studies have shown that higher concentration of Hcy in PD is related to long-term administration of L-DOPA (Lamberti et al., 2005; Ibrahimagic et al., 2016). Therapeutic approaches against ~~hyperhomocysteinemia~~hyperhomocysteinaemia include COMT inhibition~~, -~~ reducing S-adenosylhomocysteine by blocking COMT's O-methylation of L-DOPA~~, -~~ and in turn lowering its subproduct~~, Hcy~~ Hcy, and the risk of associated co-morbidities and neurodegenerative burden (Paul and Borah, 2016~~). -~~; Zoccolella et al., 2006b),

To investigate COMT inhibition in DA replacement therapy in a preclinical setting, we used ~~a~~vesicular monoamine transporter 2-deficient (VMAT2-LO) mice. This genetic animal model of PD ~~that~~ encompasses many of the progressive motor and non-motor symptoms described in patients, particularly in aged subjects. During our window of pharmacological intervention, from 14 to 18 months of age, these animals show gradually decreased motor function, as well as ~~the hallmark neurpathology of the disease, such as the vesicular monoamine transporter 2-deficient (VMAT2-LO) mice~~age-dependent decline of DAT expression and activity, contributing to neuronal

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degeneration in older animals (Taylor et al., 20112009). We specifically aimed to examine whether adding tolcapone to L-DOPA, with consecutively mildly-enhanced prolonged dopaminergic stimulation at bioavailable L-DOPA equivalent doses, reduces the progression of motor deficits during OFF-medication and striatal neuropathology – which is reportedly linkedthe cause for the development to dyskinesias— and motor deficits progression.. In addition, as a secondary outcome, we examineexamined whether adding this COMT inhibitorcombining tolcapone with prolonged L-DOPA treatment indeed reduces plasmatic levels of total Hcy (tHcy).–) and, possibly, contributes to an overall ameliorated condition.

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2. Materials and Methods

2.1 Animals

In total, we included 21 VMAT2-LO and 23 VMAT2-WT fourteen-month-old male mice with C57BL/6 genetic background. The VMAT2-LO line, obtained from the group of Gary Miller at Emory University, USA (Caudle et al., 2007), expresses only ~5% of the VMAT2 protein (Mooslehner et al., 2001). We single-housed all animals with food and water provided ad libitum until completion of the studies. We kept the animals on a 12-hour light/dark cycle and constant temperature (21 - 22°C) and relative humidity (30%). We weighed all mice daily and acclimated them to the experimental room before behavioral testing. We carried our studies approved by both local and federal Swiss authorities under the license n. ZH 205/2012.

2.2 Study design

We aimed to determine whether combined L-DOPA + tolcapone administration, in comparison to single drug administration, had an OFF-medication (i.e. after drug wash-out) beneficial effect on *functional* behavioral and histological disease hallmarks. Thus, we intended to initiate interventions after the onset of the majority of motor symptoms (*functional* behavioral markers) and when dopamine transporter (DAT, *functional* histological marker) levels were already affected, but preceding the age when neurodegeneration was first reported, indicated by tyrosine hydroxylase cell loss in the substantia nigra. We based the temporal window and duration of the treatments on the disease dynamics reported earlier for this model (Taylor et al., 2011).

At 14 months of age, we began the chronic 17-week intervention with one week of baseline behavioral testing followed by four identical periods consisting of 3 weeks of pharmacological treatment (ON-period) plus 1 behavioral testing week (OFF-period) (**Figure 1**). We administered drugs *per os* twice daily at lights-on and 8 hours later. After the last behavioral testing week (17th week, OFF-period, after 5.5 days wash-out of all drugs), we obtained plasma samples to quantify liver transaminases, euthanized the animals and collected their brains and livers for later evaluations.

2.3 Pharmacological interventions

We grouped all mice into four treatment sets: vehicle (NaCl 0.9%, '-V'); L-DOPA/benserazide (20.0 mg/kg: Madopar® 125 mg, Roche Pharma AG, Switzerland, '-L'), tolcapone (15.0 mg/kg: Tasmar® 100 mg, MEDA Pharma GmHB, Switzerland, '-T'); and a 1:1 solution of L-DOPA/benserazide (12.5 mg/kg) and tolcapone (15.0 mg/kg: '-LT'). We aimed for equalized concentrations of L-DOPA/benserazide at the synaptic level in both -L and -LT groups, by multiplying the ordinary L-DOPA dosage

(12.5 mg/kg) by 1.6, in order to achieve the same DA bioavailability in the -L group (20.0 mg/kg) (Bonifácio et al., 2015). Each mouse received a total of 120 doses complemented with a daily portion of wet-food (water and crushed chow) in addition to the regular chow.

2.4 Behavioral tests

We performed all tests in OFF-medication weeks (start 3.5 days after the last treatment episode), during the light period. We conducted all tests in 2 consecutive days, following the same order and at the same time of day.

To assess locomotor activity, we used the open field test (Bello et al., 2011): 4 identical plexiglas boxes (46x46x40 cm) and a video camera connected to an automated tracking system (EthoVision XT 9.0, Noldus, Germany). We habituated animals to the setup for 15 minutes prior to the testing day. During the testing trial, we allowed each animal to freely explore the arena for 30 minutes while recording spontaneous horizontal distance travelled.

We used the bar test to assess catalepsy, a syndrome often attributed to dopaminergic antagonism or failure of DA neurotransmission, and interpreted to reflect bradykinesia, rigidity and abnormal posture (de Ryck et al., 1980; Alvarez-Cervera et al., 2005; Noain et al., 2013). Briefly, we placed the animals sitting on the working bench over their hind paws and grabbing an elevated acrylic bar with their front paws. We considered a trial valid when the animal remained grabbing the bar at least 2 seconds and up to 180 seconds.

To examine motor coordination, we performed a standard mouse rotarod test (UgoBasile©, Italy) (Avale et al., 2004), set at constant speed. Three hours before the

testing session, we subjected the animals to a 10-minute training. During the testing phase, we placed the animals onto the rotating treadmill until a maximum time of 180 seconds.

2.5 Food intake

To examine the potential influence of feeding behavior and/or body weight on pharmacodynamics, we performed a modified food-intake test during the 15th week (ON-period) in all animals. Briefly, we removed all dry food-pellets from the cages and, for 4 days, we only presented wet-food. We weighed the cups daily and corrected the results for food evaporation.

2.6 Total homocysteine quantification

To confirm whether tolcapone is indeed helpful in reducing Hcy levels during prolonged L-DOPA replacement, we assayed standard ELISA determinations of ~~tHcy in~~ plasma ~~of VMAT2-WT animals~~tHcy using a mouse homocysteine kit (Crystal Chem, Catalog #80444, USA) as per manufacturer's protocol. The absorbance readings from VMAT2-LO animals samples were increased up to 20-fold across all groups compared to VMAT2-WT-V animals, and therefore, considered untrustworthy given the accuracy limits of the commercial kit. VMAT2-LO mice have been reported to present increased levels of ~~cysteination~~free cysteinyl-DOPA and cysteinyl-DOPAC adducts, derivatives of ~~proteins~~the reaction of DOPA and DOPAC quinones with free cysteine (Caudle et al., 2007~~),~~). The specificity of the commercial kit to Hcy in plasma samples also containing abnormally high levels of cysteinyl isomers was not assured by the

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provider, for which levels of tHcy could not be specifically determined in this genotype (data not shown).

2.7 Liver enzyme quantification

To test whether our heaviest drug regime (-LT), as proxy of all other treatments (Longo et al., 2016), negatively affected liver function in VMAT2-LO mice, we measured plasma levels of aspartate transaminase (AST) and alanine transaminase (ALT), in VMAT2-LO-V and VMAT2-LO-LT, by standard blood test. Two samples from the VMAT2-LO-LT group were found hemolyzed at the time of analysis, and were discarded by advice of the Center for Laboratory Medicine and Pathology (USZ).

2.8 Full-body perfusion

For histological analyses, we sacrificed all animals by trans-cardiac perfusion at 18 months of age (exsanguination using ice-cold phosphate-buffered-saline followed by perfusion of freshly prepared ice-cold 4% paraformaldehyde (Sigma Aldrich). Immediately after fixation, we harvested the brains, post-fixed, dehydrated, quick-froze in dry ice and stored them at -80°C. Later full-brain sectioning was done at 30 µm thickness in freezing-staged microtome. Due to freezing issues with the microtome, we discarded two VMAT2-LO-V brains from further analysis.

2.9 Immunohistochemistry, microscopy and color-density quantification

We included every fourth coronal section from the rostral portion of the striatum. Briefly, after washing the sections in tris-buffered-saline, we blocked endogenous

peroxidase activity by incubating sections in 2% H_2O_2 for 30 min. We incubated free-floating sections with a monoclonal rat anti-DAT antibody (#MAB369; Merck; 1:500) overnight at 4°C. Next, we incubated the sections in a biotinylated goat anti-rat secondary antibody (#AB97057, Abcam, 1:1000) for 2 hours at RT followed by avidin-biotin complex for 1.5 hours at RT. We developed sections with 0.025% 3,3′-diaminobenzidine (DAB) and 0.05% H_2O_2 in tris-buffered-saline.

Using an Axio Imager M2 microscope (Karl Zeiss, Jena, Germany), we acquired images from the coronal section with the highest signal intensity for quantification of DAT stained terminals (Ruifrok and Johnston, 2001). We obtained full-section images with a 2.5x objective, and further analyzed them with ImageJ (version 1.47j, National Institutes of Health, USA). Briefly, we processed all images using the hematoxylin-3,3′-diaminobenzidine vector, in order to obtain the DAB exclusive quantification panel. From each section, the signal estimate is the average color intensity, which is proportional to the concentration of the stain, of bilateral measurements in the striatum. All values were corrected for area and background-noise, the last by subtracting the intensity in the cortex. As per the Lambert-Beer law, we calculated DAT optical density (OD, p.d.u.) according to the formula *OD= log(255/ mean_intensity_striatum) – log(255/ mean_intensity_cortex)*.

2.10 Statistics

We conducted nested 2-way ANOVA to compare the effects of all pharmacological interventions on performances in open field test, bar test and rotarod, on the last OFF-period of the protocol (**Figure 1**, 17th week, treatment(genotype)). Levene's test for equality of variances was found to be violated for the time of immobility in the bar test

($F(7,36)=3.95$, $^{**}p=.003$), and for latency to fall in the rotarod test ($F(7,36) = 15.86$, $^{***}p<.001$), therefore data was ranked. On other instances, t-statistics not assuming homogeneity of variance were corrected with Welch's test. Error bars in the figures represent ±SEM. Raw data will be made available upon reasonable request.

3. Results

3.1 Improved progression of bradykinesia and postural stability scores OFF-medication in VMAT2-LO mice chronically co-administered with L-DOPA and tolcapone

Determining the effect of different chronic therapeutic interventions on motor behavior after washing out medication is a suitable instrument for assessing disease-modifying effects. To determine the progression of motor deficits in pharmacologically intervened VMAT2-LO mice and VMAT2-WT controls, we used the open field, bar and rotarod tests, with a particular emphasis on so-called axial symptoms like postural impairments, as such signs are considered particularly important for measuring motor progression (Schreiner et al., 2019).

At baseline, VMAT2-LO mice performed significantly worse in all three motor tests as compared to VMAT2-WT controls, that is impaired locomotor activity (unpaired $t(42)=6.03$, $^{***}p<.001$; **Figure 2a**), increased time of immobility (Welch's $t(20)=5.23$, $^{***}p<.001$; **Figure 2b**) and poorer motor coordination (unpaired $t(42)=4.17$, $^{***}p<.001$; **Figure 2c**).

None of the pharmacological regimes counteracted the motor deficits in VMAT2-LO mice on the last assessment week, evidenced by impaired OFF-medication performance compared to VMAT2-WT animals in all tests. In the open field,

collectively, VMAT2-LO showed reduced locomotor activity compared to VMAT2-WT (nested 2-way ANOVA, treatment(genotype), $F(4,36)=10.43$, ***$p<.001$, **Figure 2d**) despite all treatments. For catalepsy or immobility measures, VMAT2-LO animals maintained significantly longer latency for initiation of movement than VMAT2-WT controls (nested 2-way ANOVA of ranked cases, treatment(genotype), $F(4,36)=23.06$, ***$p<.001$, **Figure 2e**), however non-vehicle subjects presented slightly better marks compared to vehicle animals within each genotype. Concerning motor coordination, symptoms in VMAT2-LO remained far from VMAT2-WT in the last testing week (nested 2-way ANOVA of ranked cases, treatment(genotype), $F(4,36)=34.45$, ***$p<.001$, **Figure 2f**). Adding tolcapone to L-DOPA did not significantly changed performance when compared to -L-DOPA alone or -vehicle treated VMAT2-LO animals (Tukey post hoc comparison, L*LT and V*LT, $p>.999$).

The results from the last behavioral assessment poorly reflect the longitudinal perspective of 17-weeks of progressive behavior: groups present high variability and low statistical power at the last protocol-week as well as in the other OFF-periods (5^{th}, 9^{th} and 13^{th} weeks). When analyzing the global motor *progression*, from baseline to endpoint, in relation to the treatments (comparison of slopes of linear robust-fittings from each subject's scores), the progressive worsening in the bar test performance experienced by VMAT2-LO-LT mice appeared mitigated when compared to VMAT2-LO-L, (unpaired $t(8.43)=4.24$, **$p=.003$, **Figure 2k**). Alternatively, the improved performance in the bar test could be explained by prolonged efficacy of dopaminergic stimulation by the addition of tolcapone, with a clinical effect even days after the last medication intake. Apart from this isolated finding, progression of the other motor variables was similar between different treatments, again with high intra- and inter-variability (**Figure 2g-l**).

3.2 VMAT2-LO mice co-administered with L-DOPA and tolcapone had rescued striatal dopamine transporter levels compared to vehicle-treated transgenic mice

DAT quantification in striatal tissue is a widely established marker of functional integrity of the DA system and was previously used to describe disease progression in VMAT2-LO mice (Caudle et al., 2007; Taylor et al., 2009). We estimated DAT reactivity in the striatum (**Figure 3a**) of VMAT2-WT-V and all VMAT2-LO groups. VMAT2-LO-V mice presented reduced DAT signal strength compared to VMAT2-WT-V controls (Welch's $t(29.86)=2.43$, $^{*}p=.022$, **Figure 3b**), consistent with an ongoing functional decay in integrity of the striatal dopaminergic system in the mutants (Caudle et al., 2007). Remarkably, VMAT2-LO-LT mice presented a partially rescued level of DAT immunoreactivity ~~compared to VMAT2-LO-V (one-way~~(Welch's ANOVA, ~~Brown-Forsythe *FW*(3,69)=4.83, **~~31.43)=9.185, ***p=.004~~<.001, **Figure 3c, d**), indicating a significantly less deteriorated histopathological status ~~(Tukey~~compared to VMAT2-LO-V (Games-Howell's post hoc comparison, V*LT, ~~**~~***p=.~~004~~033, **Figure 3d**). The observations between VMAT2-LO-V and VMAT2-LO-T (p~~>.999~~=.994), and VMAT2-LO-V and VMAT2-LO-L ~~and VMAT2-LO-LT~~ mice (p=.~~142~~450) were statistically similar (**Figure 3d**). VMAT2-WT are statistically equal across all groups (Kruskal-Wallis test, $H(3)=.098$, $p=.992$, **Figure 3e, f**).

3.3 Improved immobility scores are associated with rescued neuropathological status in VMAT2-LO mice treated with L-DOPA and tolcapone

There is an association between progression of motors and non-motor symptoms and neuropathological status in both PD patients and animal models (Giasson et al., 2002;

313 Braak et al., 2003). To determine whether the immunoreactivity level of a functional
314 integrity striatal marker predicts well motor ability in the bar test, we calculated a
315 Pearson correlation between striatal DAT optical density and the bradykinesia
316 progression index (slope over the scores of all time points for the time of immobility in
317 the bar test) during OFF-medication in VMAT2-LO mice (**Figure 4a**). We found a
318 significant correlation (Pearson's, $r(17)=-0.50$, $^{*}p=.015$), which could suggest an
319 association between the effect of tolcapone-mediated steadier bioavailability of L-
320 DOPA and improved motor performance progression in this model. This link was
321 visually confirmed in an exploratory group-based clustering across the correlated
322 factors, including all mutant groups and the VMAT2-WT-V group as reference (**Figure**
323 **4b**). This figure reveals that the VMAT2-LO-LT individuals (green) are closer
324 positioned to the VMAT2-WT-V (black) centroid, overall reflecting that combined
325 treatment with tolcapone provides behavioral and immunohistochemical findings that
326 are closest to those of healthy animals.

327

328 *3.4 Food intake upon different treatments in VMAT2-LO mice*

329 VMAT2-LO mice present a distinct phenotype compared to VMAT2-WT littermates,
330 thus, we characterized the feeding behavior of all animals in ON-medication. We
331 assayed a tightly-controlled protocol and found a treatment-independent food-intake
332 behavior in VMAT2-LO mice in respect to VMAT2-WT animals (two-way ANOVA,
333 $F(3,37)=0.19$, genotype*treatment interaction, $p=.906$, **Figure 5a**), indicating that 15
334 weeks of different pharmacological regimes did not differentially affect feeding
335 behavior and, possibly, feeding-related confounders on behavior. However, VMAT2-
336 LO animals appeared to eat significantly more than VMAT2-WT controls in respect to

their body weight (two-way ANOVA, $F(1,37)=18.36$, genotype main effect, $^{***}p<.001$, **Figure 5a**).

3.5 Effect of chronic combined administration of L-DOPA and tolcapone on plasma levels of tHcy and hepatic enzymes

In agreement with previous evidence (Miller et al., 2003), we found that administration of L-DOPA increased the levels of tHcy in plasma and pushed them over the maximum standard concentration (one-way ANOVA $F(3,18)=3.49$, $^{*}p=.037$, **Figure 5b**) when compared to vehicle animals (Tukey post hoc test, $^{*}p=.038$). As expected, vehicle and tolcapone administration alone did not result in increased tHcy generation. Notably, stabilizing the bioavailability of L-DOPA with tolcapone appeared to restore tHcy plasma levels to normal values (Tukey post hoc test, $p=.865$, **Figure 5b**). Normal liver function was assessed on the last week of treatment by measuring AST and ALT plasma levels from blood samples of VMAT2-LO-V and VMAT2-LO-LT, the last being considered the group with the heaviest drug regime. We found no differences in ALT (unpaired $t(7)=1.49$, $p=.179$, **Figure 5c**) and AST levels (unpaired $t(7)=0.40$, $p=.703$, **Figure 5c**), nor in the AST/ALT ratio (unpaired $t(7)=0.98$, $p=.359$, **Figure 5d**), indicating no detrimental effect of chronic drug interventions at the assayed dosages on their hepatic functionality.

4. Discussion

Whether a more continuous dopaminergic stimulation, in general, and via COMT inhibition, in particular, may reduce L-DOPA-induced motor complications has been a matter of debate, despite some important high-impact publications (Stocchi and

Olanow, 2004). Available studies in human PD patients and rodent PD models could not unambiguously confirm such an effect (Katsaiti and Nixon, 2018). In particular, the STRIDE-PD study applying entacapone and L-DOPA in PD patients did not reveal a disease-modifying effect, but this negative result was hypothesized to be linked to higher L-DOPA equivalents in the entacapone group (Stocchi et al., 2010). Thus, it is conceivable that a beneficial effect from continuous dopaminergic stimulation by COMT inhibition has been masked by higher L-DOPA concentrations at the receptor site (Picconi et al., 2008). Apart from DA imbalance at the effector site, no studies are available on the long-term histopathological effects of such a strategy, i.e. whether more continuous dopaminergic stimulation via COMT inhibition reduces neuropathological findings in PD subjects.

From a behavioral point of view, we consider the present study as negative, as the main hypothesis – is adding a COMT inhibitor to pulsatile L-DOPA treatment neuroprotective? – could not be fully confirmed. In this line, the improved outcome on the bar test could merely reflect a prolonged efficacy of dopaminergic replacement with COMT inhibition rather than a disease-modifying effect, while other behavioral outcomes were similar between treatment groups. On the other hand, on an immunohistochemistry level, we observed rescued DAT levels in VMAT2-LO-LT mice, and no differences between vehicle- and L-DOPA- or tolcapone-treated animals. This result corroborates previous work in this animal model, finding no change in DAT expression after L-DOPA subchronic (what is this?) administration, although at an earlier age (Reveron et al., 2002). In contrast, DAT staining presented a statistical trend towards higher DAT reactivity in VMAT2-LO-LT when compared to VMAT2-LO-L.~~was statistically similar between VMAT2-LO-L and VMAT2-LO-LT, yet this lack of a difference might be explained by insufficient power.~~ Taken together, the behavioral

386 and immunohistochemical results might suggest that treating PD animals with L-
387 DOPA+tolcapone improves their functional prognosis and reduces signs of
388 neurodegeneration.

389 The main limitation of our study is the lack of assessment of multiple markers of
390 neurodegeneration upon treatments. This shortcoming is related to the treatment
391 window chosen for the study. While tyrosine hydroxilate neurons, likely the gold
392 standard marker of dopaminergic system integrity, are reported to die at a later stage
393 in VMAT2-LO animals (Taylor et al., 2011), altered synuclein deposition is found since
394 early age in this model (own unpublished data). This temporal disparity between the
395 two most conventional neurodegeneration markers in PD, rendered DAT the
396 intermediate outcome of choice. Rescued DAT levels by combined treatment,
397 however, is a pertinent outcome pointing to future reduced neurodegeneration in
398 VMAT2-LO mice. This is based not only on the fact that DAT reflects progressive
399 damage to the dopaminergic system, but also that it is fundamentally involved in the
400 profound neurochemical alterations that precede nigral degeneration in this model
401 (Miller et al., 1997; Caudle et al., 2007). The “dying back” hypothesis proposes that
402 decreased DAT function and expression might stand as a reliable proxy for loss of
403 striatal dopaminergic projections, and most importantly, as indicator of future damage
404 to the dopaminergic perikarya in the substantia nigra (Burke and O’Malley, 2013).
405 Interestingly, the deterioration of DAT expression and activity seen at the time of
406 sacrifice in VMAT2-LO-V mice is, reportedly, not resulting from loss of fiber density
407 (and concomitant cell death) nor alterations in DAT expression in the midbrain of these
408 animals (Caudle et al., 2007). ~~Lifetime intraneuronal compensatory mechanisms and~~
409 ~~interaction with DA quinones and reactive oxygen species may explain the decrease~~

in DAT observed in aged vehicle-treated animals (Whitehead et al., 2001). Add a limitation re the VMAT2 model, see R1?

One possible read-out of this study, therefore, is that inhibiting COMT with tolcapone in conjunction with chronic L-DOPA treatment, thus preventing DA fluctuations in the brain, might reduce DAT decline and, consequently, future nigrostriatal neurodegeneration. The behavioral outcome could partially confirm this hypothesis insomuch as VMAT2-LO-LT mice were the only group presenting stabilized bradykinesia and postural stability scores. Still, we cannot exclude a prolonged effect of dopaminergic stimulation at the receptor site, due to the addition of tolcapone, as discussed above. Nevertheless, the statistically significant link between behavior and immunohistochemical findings might further corroborate this hypothesis: slower deterioration of bar test performance in L-DOPA-treated groups appear correlated with preserved striatal DAT density levels, particularly the group co-administrated with the COMT inhibitor, to near the extent seen in VMAT2-WT-V group.

In relation to eventual confounders, it is worth to mention that there is no evidence of compensatory changes in VMAT2 expression (Reveron et al., 2002) and no exacerbated effect on the feeding behavior of the animals. Moreover, the present analysis was inconclusive on the tolcapone influence on tHcy levels in the transgenic animals, although tolcapone indeed reduced tHcy in VMAT2-WT-L animals. Lastly, we did not find signs of hepatotoxicity from chronic tolcapone administration, in line with early studies reporting harm of this drug only to very high administration dosages (Longo et al., 2016).

4.1 Conclusions

Altogether, despite equaling L-DOPA concentrations, this study does not undoubtedly support the assumption that adding a central COMT inhibitor to L-DOPA therapy provides a neuroprotective effect compared to L-DOPA alone. Although the results here are believed to reflect the deceleration of nigrostriatal degeneration insofar the integrity of the dopaminergic terminals is concerned, further experiments targeting a later intervention window enclosing other functional and/or histopathologic deficits in this PD model could clarify whether combining a COMT inhibitor can be neuroprotective. Such an attempt would be most welcome, as the present study – even as considered overall negative regarding the study hypothesis – produced results which at least indicate that mildly enhanced continuous dopaminergic stimulation might reduce DAT decline and, consequently, future nigrostriatal neurodegeneration.

From a clinical perspective, this finding would become relevant once there were strong clinical evidence supporting these preclinical insights. In other words, if large clinical delayed start design studies would confirm that smoothening L-DOPA fluctuations – be it with COMT inhibitors or with other strategies - decelerates the disease course, then clinical practice might shift towards adding a COMT inhibitor to L-DOPA plus benserazide/carbidopa in early PD patients. This seems particularly relevant as nowadays many neurologists shift back to initiate treatment with L-DOPA rather than dopamine agonists because of the many and potentially harmful side effects (i.e. impulse control disorders) of the latter.

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Figure legends

Figure 1. Experimental design and timeline. VMAT2 LO (n = 21) and WT (n = 23) mice were behaviorally tested at 14 months of age (baseline: BL), before starting a chronic regime of pharmacological interventions that lasted 17 weeks. Each treatment month consisted of 3 weeks of drug administration ('ON'-medication, blue rectangles), plus 1 week of behavioral testing ('OFF'-medication, grey rectangle). Each ON-medication week consisted of 5 days of twice daily per os dosaging, at light onset and 8 hours late, of saline (-V), L-DOPA (-L), tocalpone (-T) or L-DOPA+tolcapone (-LT). On the 15th ON-medication week, the food intake was assessed in all animals. After 17 weeks on interventions, the mice were sacrificed (red marking) and blood and tissue samples collected for posterior evaluations. (AST – aspartate transaminase, ALT – alanine transaminase, tHcy – total homocysteine, DAT – dopamine transporter).

Figure 2. Motor ability in response to chronic pharmacological interventions in VMAT2-LO mice. At baseline (BL), VMAT2-LO (n = 21) and VMAT2-WT control (n = 23) drug naïve mice were behaviorally tested in 3 different motor domains: (**a**) locomotion (open field test), (**b**) body rigidity (bar test), and (**c**) coordination (rotarod test). In all three tests, VMAT2-LO mice performed significantly worse than VMAT2-WT ((**a**) unpaired t(42)=6.03, ***p<.001; (**b**) Welch's t(20)=4.56, ***p<.001; (**c**) unpaired t(42)=4.17, ***p<.001). At endpoint testing, after 16 weeks of interventions, all VMAT2-LO mice presented a marked behavioral impairment in all parameters explored, as compared to VMAT2-WT controls ((**d**) nested 2-way ANOVA, treatment(genotype), $F(4,36)=10.43$, $^{***}p<.001$; (**e**) nested 2-way ANOVA of ranked cases, treatment(genotype), $F(4,36)=23.06$, $^{***}p<.001$; (**f**) nested 2-way ANOVA of

ranked cases, treatment(genotype), $F(4,36)=34.45$, $^{***}p<.001$). The progression of individual symptoms in VMAT2-LO mice in respect to the treatment received was analyzed in subsets: (**g, h, i**) -V *vs* -T treated VMAT2-LO animals were compared to evidence a potential stand-alone effect of the drug, aside from its effect over L-DOPA stability; (**j, k, l**) -L *vs* -LT treated VMAT2-LO animals were compared to evidence the effect of stabilizing L-DOPA bioavailability on motor progression. -V *vs* -T progressions were not significantly different (t-test on grouped slopes), while -L vs -LT progressions in the bar test differed significantly (**k**, unpaired $t(8.43)=4.24$, $^{**}p=.003$), with the latter evidencing practically no worsening of body rigidity in VMAT2-LO (slope of dark line close to 1) over a 17-week period. (V = vehicle; L = L-DOPA; T = tolcapone; LT = L-DOPA+tolcapone; number of animals: WT-V=5, WT-L=6, WT-T=6, WT-LT=6, LO-V=5, LO-L=5, LO-T=5, LO-LT=6).

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Figure 3. Preserved striatal DAT integrity in VMAT2-LO with stabilized L-DOPA bioavailability. (**a**) Analyzed area from coronal sections of striatum from VMAT2-WT and VMAT2-LO mice. (**b**) VMAT2-LO-V mice presented reduced DAT immunostaining strength compared to VMAT2-WT-V controls (Welch's $t(29.86)=2.43$, $^{*}p=.022$). (**c**) Individual examples of DAT reactivity in striatum of -V, -L, -T and –LT VMAT2-LO animals. (**d**) Statistical analyses of optical-density in differently treated VMAT2-LO animals show partially rescued DAT levels in VMAT2-LO-LT (one-way ANOVA, Brown-Forsythe $F(3,69)=4.83$, V*LT,$^{**}p=.004$). (V = vehicle; L = L-DOPA; T = tolcapone; LT = L-DOPA+tolcapone; number of animals: WT-V=5, LO-V=3, LO-L=5, LO-T=5, LO-LT=6).

Figure 4. Rigidity correlates with integrity of striatal dopaminergic terminals. (**a**) The slope of the robust linear fit on the time of immobility' scores of each VMAT2-LO individual over the 17-week treatment, representing the progression of bradykinesia during the complete intervention (<0 = improvement, >0 = worsening), and DAT optical density, as a marker of DA striatal terminal integrity, in VMAT2-LO mice correlate significantly (Pearson's, $r(17)=-0.50$, $^{*}p=.015$, n = 19). (**b**) Exploratory group-based clustering for bradykinesia progression (<0 means improvement, >0 means worsening) and DAT optical density levels for VMAT2-WT-V and VMAT2-LO-V, -L, -T and -LT groups. (V = vehicle; L = L-DOPA; T = tolcapone; LT = L-DOPA+tolcapone; number of animals: WT-V=5, LO-V=3, LO-L=5, LO-T=5, LO-LT=6).

Figure 5. Effects on food intake, tHcy plasma levels and hepatic enzymes. (**a**) Food intake was evaluated on protocol-week 15 for VMAT2-LO (n = 21) and VMAT2-WT (n = 23) mice. Ingested food per body weight was calculated for each group: no differences between treatments within genotypes were observed (two-way ANOVA, $F(3,37)=0.19$, genotype*treatment interaction, $p=.906$). However, when pulling together all animals of the same genotype, regardless of the treatment, VMAT2-LO animals show increased feeding behavior (two-way ANOVA, $F(1,37)=18.36$, genotype main effect, $^{***}p<.001$) when compared to VMAT2-WT mice. Number of animals: WT-V=5, WT-L=6, WT-T=6, WT-LT=6, LO-V=5, LO-L=5, LO-T=5, LO-LT=6. (**b**) Increased tHcy plasma levels observed upon L-DOPA treatment were normalized by combined L-DOPA+tolcapone (one-way ANOVA $F(3,18)=3.49$, $^{*}p=.037$; Tuckey multiple-comparisons: V*L, $^{*}p=.038$; V*LT , $p=.865$). Shadow indicates normal plasma tHcy levels (< 6 µmol/L) in male C57BL/6J mice. Number of animals: WT-V=5, WT-L=6, WT-T=6, WT-LT=6. (**c**) Alanine transaminase (ALT) and aspartate transaminase

762 (AST), marker enzymes of functional liver integrity, were determined in -V and –LT
763 VMAT2-LO mice. No differences were observed between treatments (ALT, V*LT,
764 unpaired $t(7)=1.49$, $p=.179$; AST, V*LT, unpaired $t(7)=0.40$, $p=.703$). Number of
765 animals: LO-V=5, LO-LT=4. (**d**) The ratio of AST/ALT was determined to also be
766 normal (AST/ALT ratio, V*LT, unpaired $t(7)=0.98$, $p=.359$). V = vehicle; L = L-DOPA;
767 T = tolcapone; LT = L-DOPA+tolcapone.